ORIGINAL ARTICLE

Basic Study

Transient receptor potential vanilloid 1-immunoreactive signals in murine enteric glial cells

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Abstract

AIM
To investigate the possible involvement of transient receptor potential vanilloid 1 (TRPV1) in maturation of enteric glial cells (EGCs).

METHODS
Immunohistochemical and immunocytochemical techniques were used to analyze EGC markers in myenteric plexus (MP) as well as cultured MP cells and EGCs using TRPV1 knockout (KO) mice.

RESULTS
We detected TRPV1-immunoreactive signals in EGC in the MP of wild-type (WT) but not KO mice. Expression of glial fibrillary acidic protein (GFAP) immunoreactive signals was lower at postnatal day (PD) 6 in KO mice, though the difference was not clear at PD 13 and PD 21. When MP cells were isolated and cultured from isolated longitudinal muscle-MP preparation from WT and KO mice, the yield of KO EGC was lower than that of WT EGC, while the yield of KO and WT smooth muscle cells showed no difference. Addition of BCTC, a TRPV1 antagonist, to enriched EGC culture resulted in a decrease in the protein ratio of GFAP to S100B, another EGC/astrocyte-specific marker.

CONCLUSION
These results address the possibility that TRPV1 may be involved in the maturation of EGC, though further studies are necessary to validate this possibility.

Key words: Enteric glial cells; Enteric nervous system; Glial fibrillary acidic protein; S100B; Smooth muscle cells

Core tip: We report that immunosignals of glial fibrillary acidic protein (GFAP) in myenteric ganglia in transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice are weaker than in wild-type mice in the early postnatal period, suggesting the possibility that the maturation of enteric glial cells (EGCs) might be retarded at least temporally in TRPV1 KO mice. Accordingly, in *in vitro* culture of isolated myenteric plexus cells/EGCs suggest that GFAP expression is affected by gene KO and an antagonist to TRPV1. The expression and function of TRPV1 in EGC merits further investigation.

INTRODUCTION
The enteric nervous system (ENS), an integrative neuronal network that resides within the gut wall, autonomously controls gastrointestinal (GI) motility, secretion and blood flow without major inputs from the brain[1,2]. The ENS is composed of two main cell types, neurons and enteric glial cells (EGC), the latter being several fold more abundant than neurons[3-5]. EGC share many phenotypical features with astrocytes, and were long believed to function mainly as support cells for neurons. However, emerging evidence has elucidated their regulatory role in a wide array of GI physiological aznd pathophysiological processes[6], including neurotransmission[7,8], motility[9-11], and inflammation[8], as well as in secretory/absorptive[12,13], barrier[8,14-16] and repair[17] functions of the intestinal epithelium and host defense against pathogens[18].

Transient receptor potential vanilloid receptor 1 (TRPV1) is a nonselective cation channel activated by exogenous plant-derived vanilloid compounds such as capsaicin and resiniferatoxin, as well as by endogenous membrane-derived lipid endocannabinoids such as anandamine, 2-arachidonoyl-glycerol and N-arachidonoyl-dopamine[19]. Moreover, TRPV1 is known to be a transducer channel activated by high temperature, low pH and mechanical/osmotic stimuli. Although attention has been directed mainly to sensory neurons as the site of TRPV1 localization, TRPV1 expression has been detected in non-neuronal tissues/cells, including keratinocytes of the epidermis, bladder urothelium, smooth muscles, liver, polymorphonuclear granulocytes, mast cells and macrophages[19].

TRPV1 has been reported to be present in astrocytes in brain[20], spinal cord[21] and retina[22], and possibly to be involved in glial activation[23], cell migration[24], amyloid-β-induced inflammation[25] and traumatic brain injury[26]. However, it is unknown whether TRPV1 is present and functional in enteric glia. In the present study, using TRPV1-deficient [knockout (KO)] mice and an acid-ethanol fixation protocol, specific TRPV1-immunoreactive (TRPV1-IR) signal was detected in wild-type (WT) EGC. In addition, the possible involvement of TRPV1 in the differentiation of EGC was investigated.

MATERIALS AND METHODS
Antibodies
Details of the primary antibodies used in the present study are shown in Table 1. The specificity of anti-TRPV1 antibodies is presented in Supplementary Figures S1 and S2. The secondary antibodies used were FITC-labeled donkey anti-mouse IgG antibody and Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, United States) for intestinal tissues and Alexa488-conjugated goat anti-mouse antibody and Alexa568-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, United States) for isolated longitudinal muscle layer-myenteric plexus (LM-MP) and cultured cells.

Animals
C57Bl/6 mice were from Charles River Laboratories Japan Inc. (Kanagawa, Japan). TRPV1-deficient (B6.129X1-Trpv1<tm1Jul>/J) mice originally obtained from Jackson Laboratories (Bar Harbor, ME, United States) were maintained at Charles River Laboratories Japan Inc. and transported to the animal facilities of Tsumura Laboratories on gestational day 14 (dams, 1

Table 1 Summary of the primary antibodies used in this study

Antibody	Provider	Source and type	Product/clone number	Immunogen	Usage
TRPV1	Bios	Rabbit polyclonal	bs-1931R	a.a.825-835 of human TRPV1 (EDAEVFKDSMAPGEK)	Figures 1-3, Suppl. Figure 1
	LifeSpan	Mouse monoclonal	LS-C122800	a.a. 819-835 of rat TRPV1 (CGSLKPEDAEVFKDSMVPGEK)	Figure 6, Figure 7, Suppl. Figure 1
GFAP	BD	Mouse monoclonal	cocktail of 4A11, 1B4 and 2E1	cow spinal cord homogenate (4A11, 1B4) or human /bovine GFAP (2E1)	Figures 1-3, Suppl. Figure 1
	DAKO	Rabbit polyclonal	Z0344	isolated GFAP from cow spinal cord	Figure 6, Figure 7
	CST	Mouse monoclonal	GA5	isolated GFAP from pig spinal cord	Suppl. Figure 2
PGP9.4	Abcam	Guinea pig serum	ab10410	a.a. 175-191 of human PGP9.5 (GASSEDTLLKDAAKVCL)	Figure 6, Figure 7
S100β	Proteintech	Rabbit polyclonal	15146-1-AP	recombinant human S100β	Figure 7, Figure 8, Suppl. Figure 3
αSMA	Novus	Rabbit monoclonal	E184	synthetic peptides corresponding to N-terminus (human)	Suppl. Figure 2

The negative controls of immunostaining performed in this study are shown in Supplementary Figure S6. Corresponding sequence of mouse TRPV1: TGSLKPEDAEVFKDSMAPGEK. The amino acid underlined is different from rat and the amino acid double-underlined is different from human. TRPV1 KO mice used in the present study preserved this sequence but the signal of immunohistochemistry in KO mice is faint, even in case detected, compared to that in WT mice, in the same staining and signal development protocols on the same slide on which the specimens from KO and WT mice were mounted together. Similar observations have been reported by Yamada *et al*, J Histochem Cytochem 2009; 57: 277-287.

dam per cage) or at the age of 7 wk (adult males, 4 mice per cage). The animals were allowed free access to water and standard laboratory food, and were housed at a temperature of 23 ± 2 ℃ with relative humidity of 55% ± 10%, and a 12:12-h light/dark cycle (with lights on from 07: 00 to 19: 00 daily).

All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Tsumura & Co. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sample collection
For the preliminary screening, 2 TRPV1-deficient (KO) and 2 C57/Bl6 WT dams were used. A total of 2 pups (each pup isolated from different dams) were randomly selected and sacrificed at each time point for both the KO and WT groups. Samples were collected on the day of birth (PD 0) and at around postnatal day 10 (PD 10-12), 20 (PD 20-21), 30 (PD 30-33), 60 (PD 61-62) and 90 (PD 91-92). After anesthesia with isoflurane, animals were transcardially perfused with ice-cold normal saline followed by ice-cold acid-ethanol solution (a mixed solution of ethanol and acetic acid at a ratio of 20:1 v/v) to fix the tissues. Large and small intestines (LI and SI, respectively) were dissected and further fixed in acid-ethanol solution overnight at 4 ℃, then cryoprotected and embedded in O.C.T. Compound (Sakura Finetech, Tokyo, Japan) for frozen sectioning according to standard procedures. For the confirmation analysis, 6 KO and 6 WT dams were used. A total of 6 pups (each pup isolated from different dams) were sacrificed at PD 6, PD 13 and PD 21. Intestinal tissue samples were collected as described above.

Immunohistochemistry of intestinal tissue sections
Ten μm-thick frozen sections were incubated in phosphate-buffered saline (PBS) for 10 min at room temperature, and then incubated with a mixture of primary antibodies overnight at 4 ℃. After a thorough wash with PBS, sections were incubated with a mixture of secondary antibodies with nuclear counterstain (TO-PRO3; Molecular Probes) for 1 h at room temperature, washed and finally mounted with Vectashield (Vector, Burlingame, CA, United States). Sections were observed and digital images were recorded with a confocal laser scanning microscope (C-1; Nikon, Tokyo, Japan).

IHC of whole-mount preparation of LM-MP
LI segments were isolated from 5-wk-old mice and the LM-MP was peeled off. The peeled LM-MPs were stretched taut, pinned flat to a silicone ring and fixed with ice-cold acetone for 30 min. After fixation, each preparation was washed 3 times for 10 min each in PBS. The preparations were placed in Superblock (Thermo Fischer Scientific, Rockford, IL, United States) containing 0.3% Triton X-100 overnight at 4 ℃. The preparations were then placed in a mixture of primary antibodies diluted in antibody diluent (DAKO Japan, Tokyo, Japan) overnight at 4 ℃. After removal from the primary antibody, the tissues were rinsed 3 times for 10 min per rinse with PBS and then incubated with a mixture of the relevant secondary antibodies overnight at 4 ℃. After a final set of rinses, the preparations were mounted on microslides and coverslipped with Prolong Gold antifade reagent (Molecular Probes). The slides were observed using confocal laser microscopy FV-100D (Olympus, Tokyo, Japan).

Co-culture of myenteric plexus cells and smooth muscle cells

We prepared MPC/SMC mixture from SI, because a far smaller number of MPC/SMC were obtained from LM-MP of LI, presumably due to the short length of the LI tract and inefficient cellular liberation from the tissue. SI segments were isolated from 5-wk-old WT and KO mice, and the LM-MP was peeled off. LM-MP was digested in digestion buffer containing 0.1% type II collagenase and 0.1% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, United States) at 31 ℃ for 30 min. The buffer was then replaced with new digestion buffer and the mixture incubated at 31 ℃ for an additional 30 min. The remaining tissue pieces were dissociated by mechanical shearing through micropipette tips. The cells were spun down at 200 g for 5 min and suspended in HuMedia-SG2 (Kurabo, Osaka, Japan). The cells were plated in type IV-collagen coated plates (BD Biosciences, San Jose, CA, United States). The medium was replaced on the next day, which led to discarding of almost all of the cells. The remaining attached cells, which were round and small, began to proliferate discernibly by around day 3. The cells were cultured for an additional 8 d. A representative image of the cells is shown in Supplementary Figure S3. Analysis by phase-contrast microscopy showed no apparent difference in the number and appearance of the cells between cultures derived from WT and KO mice.

Enriched EGC culture

Co-culture of myenteric plexus cells and smooth muscle cells (SMCs) was initiated as described in the previous section. On day 5 of the co-culture, the cells were trypsinized, washed and labeled with anti-NGF receptor p75 rabbit polyclonal antibody[27] for 5 min on ice. The cells were then washed and incubated with biotinylated anti-rabbit IgG antibody (BD Biosciences) for 5 min on ice. The cells were subsequently washed and mixed with magnetic beads conjugated with streptavidin. MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to enrich NGF-receptor p75-positive cells, which are derived from the neural crest. The cells were plated on poly-D-lysine-coated plate and cultured for an additional 6 d. Enriched EGC culture was performed using WT mice only, because the culture protocol has been found to be inapplicable to KO mice (the resultant culture contained only a trace number of surviving cells).

Immunocytochemistry (IHC) for enriched EGC cultures

The cells were fixed with 4% phosphate-buffered paraformaldehyde for 15 min. The cells were then washed twice with PBS and permeabilized in 0.3% Triton-100 in PBS for 15 min. After being rinsed with PBS, sections were incubated overnight in a mixture of the primary antibodies, after which the cells were incubated in a mixture of the relevant secondary antibodies (1:1000; Molecular Probes) for 60 min at room temperature. For TRPV1-staining, biotin-labeled anti-mouse IgG (BD Biosciences) and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) were used to amplify the signal intensity. Nuclei were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI; Life Technologies, Carlsbad, CA, United States). The cells were visualized, photographed and analyzed using Celaview RS100 (Olympus) or Image Xpress (Molecular Probes) cell imaging systems.

Imaging analysis for IHC

In IHC from intestinal sections, the green fluorescence of glial fibrillary acidic protein (GFAP) in MP was detected and quantitated using Image J image analysis software (version 1.40g; National Institute of Health, Bethesda, MD, United States, http://rsbweb.nih.gov/ij/)[28]. The fluorescence intensity was normalized to the circumferential length of the intestinal tract.

Imaging analysis for MPC and SMC co-culture system

The MPC and SMC co-culture system contained a large number of α smooth muscle actin (αSMA)-positive cells (*i.e.,* SMC) and a small population of GFAP+ cells and GFAP−αSMA− cells. Virtually no PGP9.5+ cells were detected. After eliminating GFAP−αSMA− cells by imaging analysis, αSMA+ SMC and GFAP+ cells were easily distinguished by DAPI fluorescence; SMC have large nuclei with weak DAPI staining and EGC have small nuclei with bright DAPI staining (Supplementary Figures S3 and S4). We counted the number of small bright nuclei and large dim nuclei separately by setting the gate for size and brightness of the nucleus image (Supplementary Figure S4). The number of eliminated cells was then counted. The percentage of GFAP+ cells and SMC was defined as the ratio of the number of the cells in the respective populations to the total number of total MPC (DAPI-stained cells).

Imaging analysis for enriched EGC culture

In enriched EGC culture, the majority of SMCs was eliminated and most cells (> 90%) were stained with anti-GFAP, anti-S100B, or both antibodies. GFAP was stained with mouse monoclonal antibody and visualized with Alexa488-conjugated anti-mouse IgG. S100B was stained with rabbit polyclonal antibody and visualized with Alexa568-conjugated anti-rabbit IgG using Celaview (Supplementary Figure S5). For quantitation of fluorescence, we used a different laser source with a different wavelength (excitation wavelengths of 488 and 568 nm, respectively) through different band path filters under different exposure settings optimal for each fluorescence. Therefore, it was not possible to directly compare the fluorescence intensity values that had been obtained using different methods and measuring rules. As a result, in this analysis, we measured the fluorescence of GFAP and
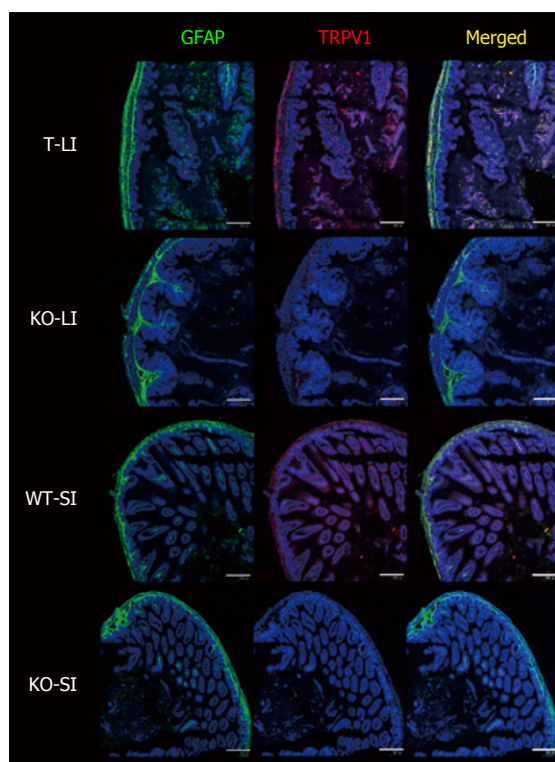
Figure 1 Double-immunostaining with GFAP and TRPV1 of small and large intestines of wild-type and transient receptor potential vanilloid 1-deficient mice. The degree of GFAP-IR signal observed in the enteric nervous system was similar in WT and KO mice. TRPV1-IR signal was observed only in WT mice and was located in a confined area of the smooth muscle layer. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 200 μm. TRPV1: Transient receptor potential vanilloid 1; SI: Small intestine; LI: Large intestine; WT: Wild-type; KO: Knockout; GFAP: Glial fibrillary acidic protein.
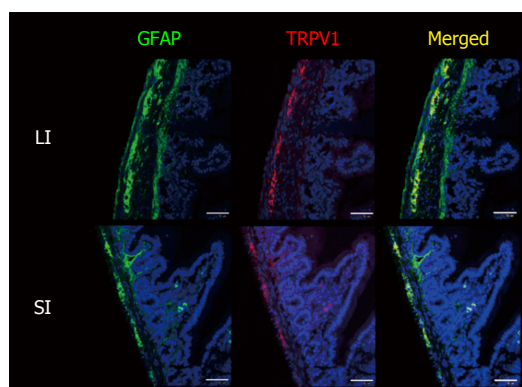

Figure 2 Enlarged view of GFAP/TRPV1-stained small intestine and large intestine of wild-type mice. Certain parts of Figure 1 were magnified. GFAP is located in the myenteric plexus, submucosal plexus and fibrous structures penetrating into the circular muscle layer and mucosal layer (in WT LI, the serosal membrane was also stained with GFAP; however, serosal membrane is known to show false-positive immunosignals with various antibodies). The location of the TRPV1-IR signal coincided with some portions of the GFAP-IR signal in the myenteric plexus. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm. GFAP: Glial fibrillary acidic protein; TRPV1: Transient receptor potential vanilloid 1; SI: Small intestine; LI: Large intestine; WT: Wild-type.
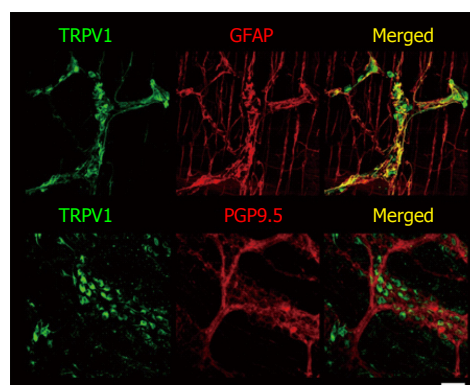

Figure 3 Double-immunostaining with TRPV1 and GFAP or PGP9.5 of large intestine longitudinal muscle layer-myenteric plexus of wild-type mice. LM-MP was isolated from 5-wk-old mice and whole-mount immunohistochemistry was performed as described in Materials and Methods. TRPV1 stained both cell bodies and fibers, while GFAP stained mainly fibers and some portions of their IR signals were co-localized. Co-localization of PGP9.5 and TRPV1-IR signals was not observed. Nuclei were stained with DAPI. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm. TRPV1: Transient receptor potential vanilloid 1; GFAP: Glial fibrillary acidic protein; LM-MP: Longitudinal muscle layer-myenteric plexus; WT: Wild-type.

S100B separately comparing the BCTC-treated wells and control wells. These cells had been isolated from a single preparation and dispensed into the wells at the same density.

Statistical analysis
Data are expressed as mean ± SEM. Unpaired Student's *t*-test was used to analyze differences between 2 groups. For comparison among 3 groups, the Dunnett test was performed. $P < 0.05$ was considered to indicate a significant difference.

RESULTS
Expression of TRPV1 and GFAP was analyzed in LI and SI, of WT and KO young adult mice, by IHC (Figure 1). While a similar level of GFAP-IR signals was detected in both WT and KO mice, TRPV1-IR signal was abolished in KO mice. Several antibodies against TRPV1, including both monoclonal and polyclonal antibodies, gave essentially the same result (2 examples of which are shown in Supplementary Figures S1 and S2). In magnified view, TRPV1-IR signals were detected in a population of GFAP+ cells (*i.e.,* EGC) mainly within the MP in both LI and SI (Figure 2). These data suggest that, at least some fractions of type Ⅰ (intraganglionic) and/or type Ⅱ (interganglionic) EGC[5] in the MP express TRPV1. The observation was further supported by high magnification IHC using isolated LM-MP. As shown in Figure 3, TRPV1-IR signal co-existed with GFAP-IR signals but not with the enteric neuron marker PGP9.5-IR.

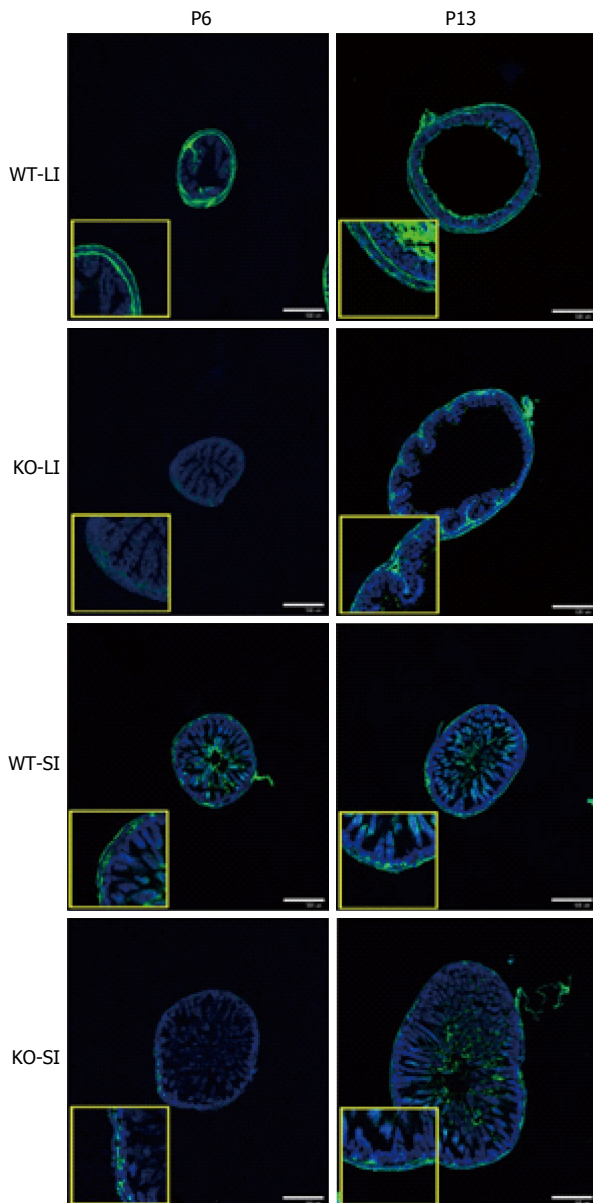To investigate the functional role of TRPV1 in the

Figure 4 Difference of glial fibrillary acidic protein immunosignals between wild-type and knockout mice (image). Large intestine (LI) and small intestine (SI) samples were isolated from WT and KO mice on postnatal day (PD) 6 and PD 13. While the GFAP-IR signal was similar in WT and KO mice on PD 13, the signal on PD 6 was weaker in KO mice than in WT mice, both in SI and LI. Nuclei were stained with TO-PRO3. Representative data of an experiment using 6 mice per each time point/group. Scale bar represents 500 μm. GFAP: Glial fibrillary acidic protein; WT: wild-type; KO: Knockout.

postnatal development of EGC, we screened a series of LI and SI samples both from WT and KO mice at various postnatal days ranging from PD 0 to PD 92 by visual inspection. Only the samples obtained at PD 5 showed an apparent difference in GFAP-IR signals between WT and KO mice. Accordingly, image analysis was used to quantitate GFAP-IR signals at PD 6, PD 13 and PD 21 in WT and KO mice (n = 6 per time point). The results revealed that GFAP-IR signals at PD 6 were significantly weaker in KO mice than in WT, both in LI and SI; however, this difference was not observed at

PD 13 nor PD 21 (Figures 4 and 5).

To obtain information about the possible influence of TRPV1 on EGC, we subsequently performed experiments using a co-culture system with MPC and SMC. Expression of GFAP and αSMA protein started at around culture day 5. At the end of the culture period, as described in Materials and Methods, most of the cells were αSMA+ SMC and the remainder was GFAP+ cells and αSMA- GFAP- cells. The results are in good accordance with those of previous studies[29-32].

Using the same digestion solution and preparation procedure as described above, cells were prepared from LM-MP of WT and KO mice simultaneously. The cells were cultured for 11 d and the number of αSMA+SMC, GFAP+ cells and αSMA-GFAP- cells was counted and represented as the ratio of the number of each cell population to the total cell number. The experiment was repeated 4 times; the results are summarized in Figure 6. The yield of GFAP+ cells was significantly lower in KO than WT mice, while the yield of SMC was not.

Enriched EGC were prepared from the 5-d co-cultures as described in the Materials and Methods, and the resultant culture contained EGC as the major cell population and a smaller population of SMC. The IHC results for the enriched EGC culture are shown in Figure 7. TRPV1-IR signal was present in GFAP+ and/or S100B+ cells but not in αSMA+ cells.

Finally, we examined the effect of the TRPV1 agonist capsaicin and the TRPV1 antagonist BCTC on the expression of glial markers in enriched EGC cultures (Figure 8). GFAP-IR signals, but not S100B-IR signals, were significantly decreased by BCTC at 3 μmol/L. Capsaicin at concentrations of 33, 100, 3000 and 10000 nmol/L had no effect (data not shown).

DISCUSSION

Many researchers have reported the presence of functional TRPV1 in the sensory nerves of the GI tract[33-36] and some have also reported its presence in intrinsic enteric nerves of the MP[37-39]. It is controversial as to whether TRPV1 exists in GI structures other than extrinsic nerves, apart from in infiltrating inflammatory cells[40,41]. The immunostaining pattern shown in the present study resembled that of previous studies[37-39], demonstrating TRPV1-IR signals in intrinsic enteric nerves of the MP of guinea pig SI and LI. Thus, to our knowledge, our study is the first and only report addressing the possible presence of TRPV1 in EGC. The specificity of the antibodies used in this study was validated using TRPV1 KO mice (Figures 1 and 2) and recombinant TRPV1-expressing cells (Supplementary Figure S2). There are several possible reasons for the apparent discrepancies between our results and those of previous studies.

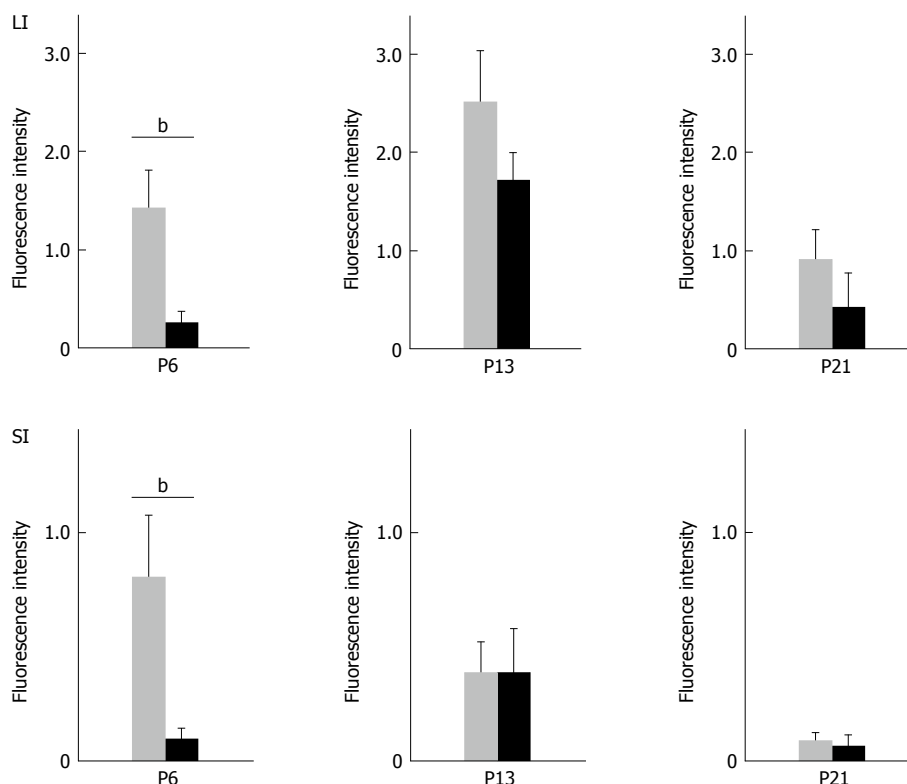Firstly, the different antibodies may specifically detect different forms of TRPV1 protein. Buckinx *et al*[39]

LI

SI

Figure 5 Difference in glial fibrillary acidic protein-IR signals between wild-type and knockout mice. The intensity of GFAP-IR signal for WT and KO mice on postnatal day (PD) 6, PD 13 and PD 21 was quantitated by imaging analysis as described in Materials and Methods. The amount of GFAP-IR fluorescence was normalized to the circumferential length of the intestinal tract. Data represent mean ± SEM, *n* = 5-6. [b]$P < 0.01$. GFAP-IR: Glial fibrillary acidic protein; WT: Wild-type; KO: Knockout.

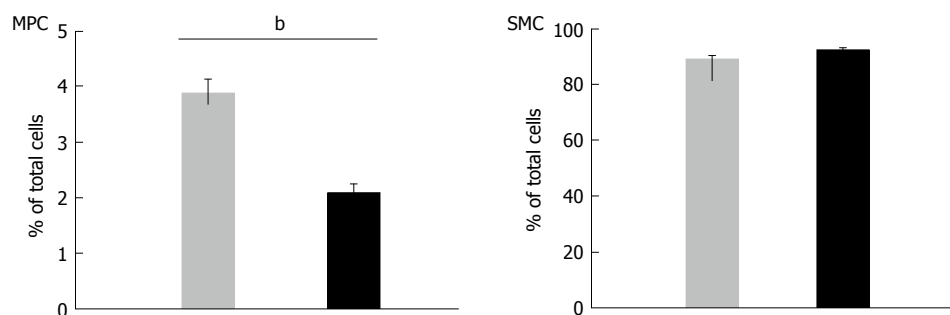MPC   SMC

Figure 6 Specific decrease in the number of myenteric plexus cells in knockout mice. The cells were prepared from longitudinal muscle layer-myenteric plexus (LM-MP) of wild-type (WT) and KO mice simultaneously, using the same digestion solution in a single preparation. After 11-d culture, the numbers of α smooth muscle actin (αSMA)+ smooth muscle cells (SMCs), glial fibrillary acidic protein (GFAP)[+] cells and αSMA[-]GFAP^{-} cells were determined as described in Materials and Methods. Nuclei were stained with DAPI. The percentage of GFAP^{+} cells and SMCs was calculated as the ratio of the number of each cell population to the number of total DAPI^{+} cells. The yield of enteric glial cells (EGCs, GFAP+ cells) in KO mice was significantly lower than that in WT mice, while the number of SMCs obtained was similar for both types of mice. Data represent mean ± SEM, *n* = 4. [b]$P < 0.01$. KO: Knockout.

have reported that a guinea pig and a rabbit antibody raised against slightly different regions of the C-terminus of mouse TRPV1 yielded different staining patterns; *i.e.,* the former stained cytosolic IR signals and the latter stained fibrous IR signals. The antibodies used in the present study appeared to stain both cell bodies and fibers. TRPV1 protein is suggested to be present and functional in the cell membranes as well as intracellular organelles such as the endoplasmic reticulum, Golgi bodies and mitochondria[42-48]. These

different staining patterns might therefore result from conformational differences between intracellular and plasmalemmal TRPV1. Differences in interacting molecules also influence epitope recognition by antibodies. It should be noted the C-terminal region of TRPV1 contains several modulatory regions, such as phosphorylation sites and binding sites for calmodulin and phosphatidylinositol 4,5-bisphosphate[49,50].

Secondly, we used acid ethanol fixation while the above-mentioned studies used methanol, Zamboni's

Figure 7 Immunostaining of transient receptor potential vanilloid 1 in enriched enteric glial cell culture. EGCs were isolated and cultured as described in Materials and Methods. The cells were labeled with antibodies to glial fibrillary acidic protein (GFAP), S100B or smooth muscle cell (SMC). The cultures contained many EGCs and a small percentage of SMCs. The location of TRPV1-IR signal coincided with that of the GFAP-IR signal and the S100B-IR signal, but not with that of the αSMA-IR signal. Nuclei were stained with DAPI. Scale bar represents 20 μm. TRPV1: Transient receptor potential vanilloid 1; EGC: Enriched enteric glial cell.

and paraformaldehyde fixation. IHC of certain glial proteins has been known to provide different results depending on the fixation procedure. For example, certain anti-GFAP antibodies were reported to detect mainly fibrous astrocytes in brain white matter after acid-alcohol fixation, while protoplastic astrocytes are detected in brain grey matter after aldehyde-based fixation[51]. Comparison of the intensity of IHC with

A GFAP

B S100B

Figure 8 **Effect of transient receptor potential vanilloid 1 agonist/antagonist on the signal ratio of glial fibrillary acidic protein-IR to S100B-IR.** Enriched enteric glial cel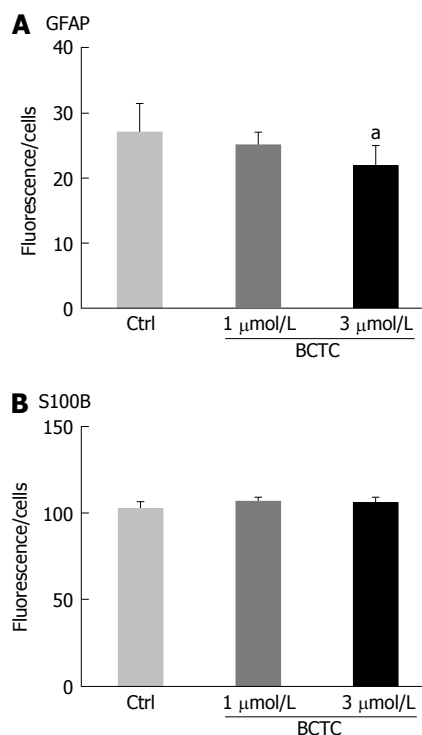ls [EGCs; prepared from day 5 myenteric plexus cells (MPCs) and smooth muscle cells (SMCs) co-culture] were plated and cultured for an additional 5 d. The TRPV1 antagonist BCTC was added and after 24 h the cells were fixed. The cells were stained with antibodies and imaging analysis was performed using a Celaview system as described in Materials and Methods. BCTC (3 μmol/L) decreased the GFAP-IR signal but the S-100B-IR signal was unchanged. Data represent mean ± SEM, *n* = 5-8. [a]*P* < 0.05. TRPV1: Transient receptor potential vanilloid 1; GFAP: Glial fibrillary acidic protein.

the amount of GFAP protein estimated by enzyme-linked immunosorbent assay has suggested that ethanol-based fixation provides better results than aldehyde-based fixation[52]. Vimentin in astrocytes and Bergmann glial fibers in cattle, rabbit and rat brain[53], and P-glycoprotein in rat brain astrocytes[54] were also reported to be detected only after ethanol-acid fixation. Along these lines, in the course of our research on EGC ontogeny, we have found that acid-ethanol fixation provides stronger and more specific IHC for GFAP in EGC of the mucosal and smooth muscle layers of SI and LI.

Thirdly, it should be noted that the present data does not exclude the possibility of the presence of TRPV1 in enteric nerves. We did not detect the co-existence of TRPV1-IR signals with PGP9.5-IR signals, but this might have been due to the relatively poor sensitivity and resolution of the immunostaining patterns of PGP9.5 in the present study. Furthermore, previous studies demonstrating the existence of TRPV1-IR signals in intrinsic myenteric neurons used antibodies to choline acetyltransferase, calbindin and calretinin[37,38]. Matsumoto *et al*[40] also reported that

TRPV1-IR did not co-localize with PGP9.5 and NeuN in cell bodies of the MP. We found immunohistochemically-stained TRPV1^{+}GFAP[-] areas in the LM-MP. Because the GFAP antibody appeared to mainly stain fibrous structures inside the cells, the TRPV1^{+}GFAP[-] area could represent EGCs. However, it is possible that these areas are contained in structures other than EGCs. Extensive research, including studies on cellular and/or intracellular TRPV1-mediated calcium mobilization in these cell types, will be needed to clarify these issues.

TRPV1 has been reported to be expressed by astrocytes in mouse spinal cord[21], in mouse, rat and human brain[20,23,55], in rat retina[22], and in *in vitro* cultured rat astrocytes[56]. Treatment with the TRPV1 agonist resiniferatoxin was reported to increase Fos expression by astrocytes in mouse brain. Furthermore, injection of capsaicin, another TRPV1 agonist, led to an increase in markers for microglia (ionized calcium-binding adapter molecule 1, Iba1) as well as astrocytes (GFAP) in the dorsal horn of the spinal cord after adjuvant-induced arthritis or partial sciatic nerve ligation[23], and in the trigeminal nucleus caudalis[57]. Treatment with TRPV1 antagonist decreased the migration of reactive astrocytes isolated from the wounded retina[24]. These data suggest that TRPV1 stimulation resulted in the activation of astrocytes and, presumably, microglia. TRPV1 in astrocytes has also been suggested to be involved in the pathogenesis and epileptogenesis of human mesial temporal lobe epilepsy[55]. These reports suggest that TRPV1 in astrocytes is functional and plays certain roles in astroglial biology.

In the present study, firstly, TRPV1 KO mice showed weaker GFAP-IR signals only at PD 6, but not at PD 13 nor PD 21. Secondly, the number of GFAP-expressing cells developed from the isolated MPC was significantly lower in TRPV1 KO mice than in WT mice. Thirdly, treatment of isolated WT MPC with the TRPV1 antagonist BCTC resulted in a decrease in the expression ratio of GFAP to S100B; the latter is another frequently-used EGC/astrocyte-specific marker[58]. S100B is a diffusible Ca^{2+}/Zn^{2+}-binding protein that is considered to be a "janus face" neurotrophin for neuron and astrocytes[59] and to act as a proinflammatory cytokine involved in gut inflammation with specific relevance to nitric oxide production[60]. It has been speculated that the intensity and differential intracellular localization of GFAP-IR and S100B-IR signals is related to the degree of differentiation and/or functional diversity of astrocytes[39,61-64]. These data suggest that TRPV1 signaling may interfere with GFAP expression in EGC, at least during a certain period of EGC maturation.

Because GFAP is widely recognized as an astrocyte differentiation marker, constituting the major intermediate filament protein of mature astrocytes[65-67], the present finding suggests that TRPV1 might be involved in the differentiation/maturation of EGC. The ENS

originates in the neural crest, which invades, proliferates and migrates within the intestinal wall until the entire bowel is colonized with enteric neural crest-derived cells (ENCDCs)[68]. After initial migration, ENCDCs differentiate into glia and neuronal subtypes and form a critical constituent for nervous system function. Although little is known about mechanisms controlling the development and differentiation of EGC, it has been suggested that Sox-10, Lgl4, ADAM22 and bone morphogenetic proteins are involved[4]. GFAP appears later at the end of the mouse embryonic stage. Cells with functions similar to those of ENCDCs exist in the bowel of adult and newborn humans and rodents[69,70]. The present data indicate that TRPV1 might be involved in the regulation of GFAP expression in *in vitro* cultured MPC prepared from young adult animals as well as *in vivo* in the early postnatal period (around PD 6). Although the present study did not examine ENCDC markers, methods similar to ours have been used to obtain enteric neural stem cells for enteric neural stem cell therapy[71,72].

The following three points, however, should be noted. Firstly, we did not examine the functions of EGC. As reported for central nervous system astrocytes, deletion or alteration of TRPV1 signaling might influence EGC function. Further intensive investigation will be needed to clarify this point. Secondly, although a normal microenvironment around EGC can compensate for the effect of TRPV1 deletion, at least until PD 10 in the case of diseased intestines (*e.g.,* due to damage of neurons, SMCs and mucosa, the effect of TRPV1 deficiency might be more severe because of a lack of compensatory signals). Along these lines, we could not obtain enriched EGC from TRPV1 KO mice as described in the Methods section. Because enriched EGC cultures contain far smaller numbers of SMC compared to unenriched MPC culture, the signals from SMC may be important for compensation. Additional studies are needed to determine the possible effects of TRPV1 deletion on diseased intestine, such as intestinal inflammation, hyperalgesia after psychological or surgical stress, and functional dysregulation (*e.g.,* dismotility, nutrient malabsorption and diarrhea/constipation). It is possible that TRPV1 plays an important role in the repair/recovery/restore stage of GI tissues. Finally, the present study suggested the possible existence of TRPV1 in EGC mainly by immunohistochemical/immunocytochemical techniques. Biochemical isolation and identification of TRPV1 protein and functional validation of TRPV1, such as through agonist-specific Ca^{2+} influx and Na^+ current, are necessary to establish the existence and function of TRPV1 in EGC.

In conclusion, a combination of IHC, immunocyto-chemistry and isolated cell culture using TRPV1 KO mice addressed the possibility that EGCs express TRPV1 and play a role in cell maturation. Further extensive studies are needed to validate this possibility.

COMMENTS

Background

The transient receptor potential vanilloid 1 channel (TRPV1) is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli, including heat, acidic condition and capsaicin. Enteric glial cells (EGCs) are one of major cell types comprising the enteric nervous system (ENS). This study explored TRPV1 expression in mouse EGCs.

Research frontiers

Although the presence of TRPV1 in astrocytes in the central nervous system has been reported, the presence in EGCs has not.

Innovations and breakthroughs

TRPV1-immunoreactive signal (TRPV1-IR) was detected in EGCs. The temporal retardation of postnatal maturation of EGCs in TRPV1 knockout mice was suggested.

Applications

The present results address possible involvement of TRPV1 in postnatal development/maturation of EGC. Dietary TRPV1 stimulation in the weaning period may affect postnatal ENS development. However, the expression and biological function of TRPV1 in EGC requires further evaluation.

Terminology

TRPV1 is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli including heat, acidic condition and various pungent materials.

Peer-review

The authors are to be commended for the work in the manuscript entitled "TRPV1-immunoreactive signals in murine EGCs". This is an interesting paper highlighting the expression profiles of TRPV1 in murine EGCs.

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